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Six molecular patterns leading to hemophilia A phenotype in 18 females from Poland

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ABSTRACT

Introduction: Female hemophilia is an intriguing rare disorder and few larger reports on its genetic etiology are available. While historically the diagnosis was satisfactorily reached by factor VIII activity assays, the clinical and potentially therapeutic heterogeneity of female hemophilia calls for comprehensive molecular diagnosis in each case. Currently, the genetic investigations are not a part of routine, state-funded, diagnostics in Poland, and thus molecular epidemiological data are missing.

Aim: We set out to perform a comprehensive genetic analysis of Polish females with hemophilia A.

Patients/methods: Eighteen females with hemophilia A (including 2 with severe and 5 with moderate hemophilia phenotype) consented for genetic diagnostics. To establish *F8* mutations, we used next-generation sequencing of a panel of genes associated with hematological disorders, standard assays for recurrent intragenic *F8* inversions and MLPA when deletions were suspected. When appropriate we also used karyotyping, genomic microarrays and X chromosome inactivation assays.

Results: While abnormally skewed X-chromosome inactivation combined with a *F8* variant on the active allele was, as expected, the most common genetic etiology, a number of other genetic scenarios were unraveled. This included: misdiagnosis (molecular diagnosis of vWd), Turner syndrome, compound heterozygosity and androgen insensitivity syndrome (a phenotypical 46,XY female with a novel androgen receptor gene mutation). We report 3 novel *F8* mutations.

Conclusion: Every case of female hemophilia warrants full genomic diagnostics, as this may change the diagnosis or reveal broader morbidity than a coagulation disorder (Turner syndrome, androgen insensitivity, or cardiovascular morbidity that we described previously in a SHAM syndrome carrier).

1. Introduction

The molecular pathogenesis of hemophilia A in males has been known for a long time and hemophilia A is a model monogenic disease, but there is a wide variability in the genetic defects in females with hemophilia [1,2]. Although skewed X chromosome inactivation (XCI) is

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believed to be the most common cause of X-linked disease in women, there are few studies and thus no reliable data on the relative frequency of skewed XCI versus various genomic events leading to loss of *F8* expression from both alleles. There might be a bias for reporting skewed XCI if genetic defects are difficult to register in a particular case. Also, severe or moderate hemophilia must be a consequence of an extremely abnormal X chromosome inactivation pattern, itself likely caused by genomic lesions of X chromosome or mutations in genes active in XCI [1–3]. Occasionally, as we previously demonstrated, the hemophilia-causative genomic defects may result in phenotypes and morbidity broader than the coagulation defect and their identification will facilitate proper management (as exemplified by a contiguous gene syndrome associated with hemophilia A that we previously described) [4–6]. Also, there is an increasing therapeutic heterogeneity and the treatment choice in special circumstances such as hemophilia in a female should be supported by exhaustive diagnostics [7,8]. Lastly, we believe every patient with rare disease deserves thorough genetic diagnostics to enable genetic counselling and several clinical recommendations advocate for genetic work-up in females with bleeding disorders [9]. Currently, genetic testing in the setting of hemophilia is not routine and state-funded in Poland. Having this in mind, we initiated a nationwide survey for genetic causes female hemophilia A to assess the prevalence of the disorder and potentially uncover novel disease-associated genomic alterations. Prior to this study, we were not aware of any previous reports on molecular causes of this rare disease in Poland apart from our previous publication [5].

2. Materials and methods

2.1. Patients

18 females, including one pair of siblings, with hemophilia A were recruited via the centers providing hemophilia services in Poland (we approached 15 pediatric and 16 adult departments). Patients characteristics are presented in the Results section. Appropriate ethical approval was obtained from the Local Ethical Board at the Medical University of Lodz (decision No RNN/238/17/KE).

2.2. DNA extraction

Genomic DNA was extracted from peripheral blood samples of 18 patients using FlexiGene DNA Kit (Qiagen, USA) and checked for quality using Qubit v.3 (Thermo Fisher Scientific, USA).

2.3. Next-generation sequencing

The Next Generation Sequencing was performed using custom designed SureSelect QXT panel (Agilent Technologies, USA) comprising 535 genes related to hematological diseases, including Hemophilia A and vWd, as well as FVFVIII deficiency, as described in our two previous publications. Briefly, the DNA libraries were prepared according to manufacturer's protocols and sequenced on a Next Seq 550 (Illumina, USA) in the process of 300 bp paired-end run. The data analyses of the target regions were performed using BWA Genome Alignment Software and the GATK Variant Caller algorithms and mapped to the human genome reference sequence GRCh37/hg19. The results were next analyzed using Variant Studio v. 3.0 (Illumina, USA) and Integrative Genomics Viewer v.2.3. The filtering criteria included coverage with at least 20 reads and allele frequency below 0.01. All filtered variants were investigated by several bioinformatics tools: SIFT, Mutation Taster, and PolyPhen-2. The pathogenicity of the revealed variants was estimated based on ClinVar, ExAC, OMIM, HGMD, Varsome and LOVD databases. The detected variants were verified by direct DNA sequencing. Standard PCR conditions were used with the primers designed using Primer 3 v. 0.4.0. Products were sequenced on ABI 3130 4-capillary sequencer (Applied Biosystems, USA) and the results were analyzed using Sequencher v. 5.0.

2.4. MLPA

Targeted copy number screening of *F8* exons was performed in the cohort using multiplex ligation-dependent probe amplification (MLPA), which is a multiplex PCR (polymerase chain reaction)-based technique in which probes hybridized to the sample DNA, are amplified using only one PCR primers pair. A simultaneous and semi-quantitative amplification is used to determine the relative copy number of each DNA sequence in a single multiplex PCR-based reaction. The commercially available SALSA MLPA P178 F8 probemix kit (MRC–Holland, the Netherlands) was used for MLPA reactions as per manufacturer's protocol. Briefly, probe mix and hybridization buffer (MRC-Holland) were added in equal amounts to 150 ng of genomic DNA followed by heat denaturation and overnight hybridization of the probes at 60 °C. Subsequently, ligation was performed and the ligation products were amplified by PCR using a 6-FAM fluorophore-labeled primer set (MRC-Holland). The different length products after PCR were separated on an automated capillary sequencer (Applied Biosystems, USA). The size and peak areas for each probe were quantified and analyzed by data analysis software (GeneMarker v2.7.0., Softgenetics, USA).

2.5. F8 intron 22 and intron 1 inversion assays - inverse shifting-polymerase chain reaction (IS-PCR)

F8 intron 22 and intron 1 inversion assays with Inverse shifting-polymerase chain reaction (IS-PCR) were all conducted as previously described in our study of 101 Polish boys with hemophilia A [10] according to a slight modification of an inverse shifting-polymerase chain reaction (IS-PCR) described originally by Rossetti et al. [11]. 2 μg of genomic DNA was digested with 20 units of *Bcl*I (ThermoFisher Scientific, USA) at 55 °C over 4 h in 50 ul. Digested DNA was isolated using phenol:chloroform:isoamyl alcohol 25:24:1 (Sigma-Aldrich). The aqueous phase was removed and isopropanol (Sigma-Aldrich, Germany) precipitated DNA were washed with 75% cold ethanol and air-dried. The restriction fragments were circularized with 400 units of T4 DNA ligase (EURx) in 25 μl at 16 °C overnight that represent templates for a standard PCR analysis. Later DNA was extract as above and the circularized DNA was used in IS-PCR. Primer sequences are shown in Supplementary Table 1. The PCR conditions were as follows: 35 cycles, each cycle consisting of 1 min of denaturation at 95 °C, 30 s for annealing at 56 °C, and 30 s at 72 °C for extension. Cycling was preceded by 95 °C for 15 min, and followed by 5 min at 72 °C. IS-PCR products were analyzed with GelRed Nucleic Acid Gel Stain (Biotium) on 2% agarose gel electrophoresis.

The Inv22 diagnostic test uses four primers (IU, 2U, 3U and ED) to permit a diagnosis of the Inv22-1 (distal pattern), Inv22-2 (proximal pattern) and normal allele. The complementary test allows further discrimination between the pairs that remained unclassified after the Inv22 diagnostic test – wildtype allele (normal) and Dup22 by gaining the 559 bp (Supplementary Table 2). The Inv1 diagnostic test uses tree primers (1-IU, 1-ID and 1-ED). IS-PCR products of 304 bp and 224 bp indicated the normal and the Inv1 allele, respectively (Supplementary Table 3).

2.6. X chromosome inactivation (XCI) assay

X chromosome inactivation analysis based on methylation specificity of restriction enzymes at polymorphic short tandem repeats (STR) in a chosen X linked marker was performed as described in our previous publications [5,12]. The two X chromosomes in human females can be distinguished from each other by using a polymorphic CAG repeat in exon 1 of AR (human androgen receptor gene) in Xq12. The assay is based on selective activity of *Hpa*II restriction endonuclease on unmethylated but not methylated DNA and remains relatively standard in

X chromosome inactivation analysis. The XCI study included 20 healthy females who had blood drawn for various diagnostic procedures and who agreed for their blood samples to be anonymized and used as reference for genomic experiments.

2.7. HpaII and RsaI digestion of DNA

Genomic DNA (100 ng) was digested with either 20 U RsaI alone or 20 U RsaI with 20 U HpaII (both enzymes with Thermo Fisher Scientific). RsaI digest genomic DNA, but does not affect fragment size, as the PCR amplified region does not contain an RsaI cutting site, so it was used for technical reasons. Both reaction was performed in a total reaction volume of 10 μl and incubated at 37 °C for 16 h. Restriction enzymes were inactivated by incubation at 65 °C for 20 min and stored at 4 °C.

2.8. PCR amplification and fragment analysis

PCR amplification was performed using 25 ng undigested DNA or 2,5 μl of *Hpa*II and *Rsa*I digested DNA, 200 μM of each dNTP (Roche), 1× reaction buffer, 0.5 U of Hot Start Taq DNA polymerase (Qiagen), 0.1 μM of each primer (forward and reverse). The forward primer was labeled with FAM on the 5′ end; AR-F (5′-TCCAGAATCTGTTCCAGAG CGTGC-3 2032′) and AR-R (5′-GCTGTGAAGGTTGCTGTTCCTCAT-3′). These primers flank a highly polymorphic CAG repeat region in exon 1 of AR. The PCR parameters were as follows: 95 °C for 15 min and 35 cycles (95 °C for 20 s, 67 °C for 20 s, 72 °C for 25 s), followed by 72 °C for 7 min. The PCR products were separated on an ABI 3130 DNA sequencing analyzer (Applied Biosystems) and analyzed by GeneMarker software v2.7.0 (SoftGenetics). The digestion process preferentially degrades activated (unmethylated) over inactivated (methylated) DNA. Undigested DNA is preferentially amplified and produces larger peak heights. The peak height values for digested DNA are normalized using peak height values for the undigested DNA for each subject. The X - inactivation ratios (X1:X2) represent the level of inactivation of the smaller compared to that of the larger allele. The X-inactivation level of each allele was calculated using peak heights with the formulas: X1 = (d1/u1)/[(d1/u1) + (d2/u2)]; X2 = (d2/u2)/[(d1/u1) + (d2/u2)]; d1 – peak height of digested DNA from the first allele and u1 – peak height of undigested DNA from the first allele; d2 – peak height of digested DNA from the second allele and u2 – peak height of undigested DNA from the second allele.

2.9. Karyotyping (G-binding)

The process of generating a karyotype was based on culturing the cells from whole blood stimulated with phytohemagglutinin (PHA). After a period of cell growth and multiplication (72 h; 37 °C, 5% CO_2), dividing cells were arrested in metaphase by addition of colchicine, which poisons the mitotic spindle. Next the cells were treated with a hypotonic solution, destroying the nuclear membrane and releasing chromosomes. Subsequently metaphase chromosomes were fixed, dropped on a glass slide, and treated with trypsin and Wright blue solution. Karyotyping was performed with use of CytoVision software and light microscope Olympus BX51.

2.10. SNP genomic microarrays

SNP microarray analysis of the extracted DNA from the peripheral blood samples was performed using the Affymetrix CytoScan HD platform (Affymetrix), which contains approximately 2.6 million markers, and provide an average resolution of the array to be one marker per 3 Kb in the targeted regions and one marker per 5 Kb in the backbone. Purified DNA was fragmented, amplified and hybridized according to the Affymetrix Cytogenetics Assay Protocol. The scanning was performed using the GeneChip Scanner 3000DX (Affymetrix) and data obtained were analyzed using the ChAS v.3.3 software (Affymetrix).

Table 1
Basic demographic and clinical data of the study population.

Current age (years-median, range)	11 (2–64)
Phenotype (severe/moderate/mild)	2/5/11
Factor VIII activity (%-median, range)	9.6 (0.5–40)
Ethnicity (Caucasians/others)	18/0
FVIII prophylactic treatment (yes/no)	1/17
Factor VIII inhibitor (yes/no)	1/17

2.11. Polish reference population

We also accessed an anonymized database of 825 female individuals that we prepared for our previous publication, whose genetic diagnostics for indications other than hemophilia or other bleeding tendency included gene panel of whole exome next-generation sequencing, and who consented for further scientific use. This cohort served for our study as a genetic reference population of Polish females. No phenotypic data is available. The details of the dataset are available in our previous publication [10].

3. Results

3.1. Patients characteristics

Table 1 presents the characteristics of females, with FVIII activity ranging from 0.5 to 40%, included in the study. VWF:Ag and VWF:RCo values were reported as normal for all patients by all referring centers.

3.2. The genetic analysis of 18 Polish females with hemophilia A yields known and novel F8 variants

We have identified likely causative *F8* gene variants (nucleotide variants or rearrangements) in all cases in our study, apart from one subject that turned out to be suffering from von Willebrand disease type 2N. All variants determined with NGS were confirmed with Sanger sequencing. We also performed intron 22 and 1 inversion analysis in all cases. No deletion mutations were found. In total in 17 females with hemophilia there were 18 pathogenic *F8* variants (on 32 X chromosomes as there was one 45,X and one 46, XY case as described below), two of these were identical (siblings). These 18 variants included 8 cases of intron 22 inversion and 9 different pathogenic nucleotide variants. Nucleotide variants were compared against variants reported in severe hemophilia B in EAHAD (European Association for Hemophilia and Allied Disorders), CHAMP (CDC Hemophilia Mutation Project) and CHAMP US databases [13]. Table 2 presents variants present in our patient population that are identical to *F8* variants already reported in hemophilia A genetic databases or our previous analysis of Polish boys with hemophilia A [10] and 3 novel variants that were previously not described.

3.3. Six different molecular patterns leading to hemophilia A phenotype in 18 Polish females

We observe 6 different molecular patterns leading to hemophilia A phenotype in 18 Polish females. These include 1) misdiagnosis, 2) carriers of one *F8* defect with marginally depressed FVIII activity and no skewed XCI, 3) 45,X karyotype associated with *F8* defect, 4) 46,XY androgen insensitivity syndrome associated with *F8* defect are described below, 5) compound heterozygosity for *F8* mutation, 6) *F8* defects associated with abnormally skewed X chromosome inactivation.

Table 2
F8 mutations in females with hemophilia A reported in EAHAD, CHAMP and CHAMP US databases or our previous study of 101 boys (normal font) and novel F8 mutations (absent in EAHAD, CHAMP and CHAMP US databases and our previous study – **in bold**).

Number of patients	HGVS coding sequence (NM_000132.3)	HGVS protein sequence (NP_000123.1)	Database phenotype	Allele frequency among female carriers in Polish reference population
1	**c.677G > A**	**p.Ser226Asn**	–	**0**
1	c.1271 + 1G > T	splice site variant	Severe	0
1	c.1812G > C	p.Trp604Cys	Severe	0
1	c.2078C > T	p.Ser693Leu	Moderate	0
1	c.4379dupA	p.Asn1460LysfsTer2	Moderate/severe	0
1	c.5405A > G	p.Tyr1802Cys	Mild/moderate	0
1	**c.6118T > C**	**p.Cys2040Arg**	–	**0**
1	**c.6509delT**	**p.Leu2170CysfsTer16**	–	**0**
2 (siblings)	c.6929C > T	p.Thr2310Ile	Mild	0

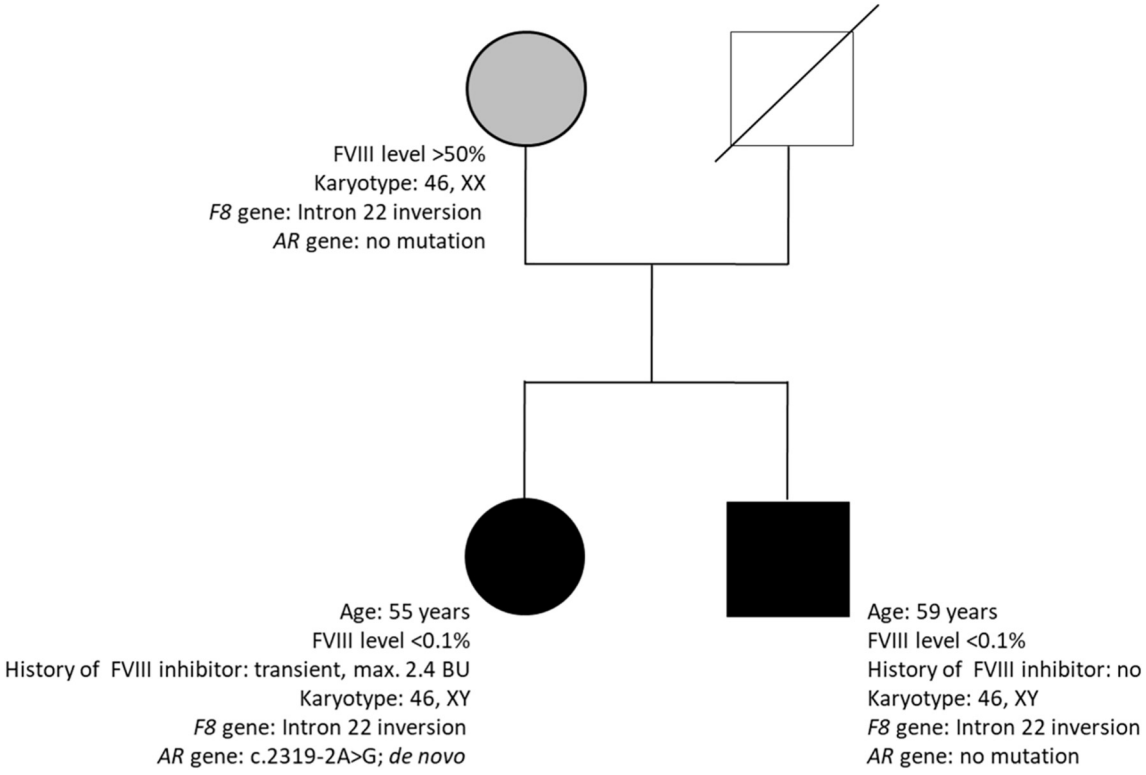

Fig. 1. Clinical and genetic characteristics and mode of inheritance in a family of a proband with androgen insensitivity syndrome and severe hemophilia A.

Table 3
The characteristics of patients in whom XCI plays a causative role (random XCI was defined as 50%: 50% to 64%: 36%, mildly skewed as 65%: 35% to 80%: 20%, and severely skewed as > 80%: 20%).

FVIII activity	F8 mutation	XCI pattern
0.5	Trp604Cys	Severely skewed
2	Intron 22 inversion	Severely skewed
3	Ser226Asn	Severely skewed
6.9	Ser693Leu	Severely skewed
7	Intron 22 inversion	Severely skewed
12.3	Intron 22 inversion	Mildly skewed
23.4	Intron 22 inversion	Mildly skewed
24.5; 34.2 (siblings)	Thr2310Ile	Severely skewed
33.1	Asn1460LysfsTer2	Mildly skewed
40	Intron 22 inversion	Mildly skewed

3.4. Misdiagnosis

In one patient with FVIII activity of 35.8% we found no sequence alterations of the *F8* genes, but instead the patient was demonstrated to be homozygotic for p.Arg854Gln *VWF* variant consistent with type 2 N von Willebrand disease. vWf antigen level was 116% (normal vWf antigen and vWf:RCO are typical for vWd type 2 with homozygotic Arg854Gln mutation [14]). No pathogenic *VWF* variants were found in the remaining females. No rare, low frequency or potentially pathogenic variants in genes associated with combined deficiency of FV and FVIII (LMAN1 and MCFD2) were found in any of the patients excluding F5F8D diagnosis.

3.5. Carriers of one F8 defect with marginally depressed FVIII activity

In 2 patients with only marginally abnormal FVIII activity (38.8% and 40%) the analysis yielded *F8* mutation on only 1 allele (intron 22 inversion) and no skewed X chromosome inactivation. This is consistent with the observation that some hemophilia carriers have FVIII activity slightly below normal levels rather than expected 50%.

3.6. Turner syndrome

One patient (FVIII activity 4.5%) was demonstrated to suffer from

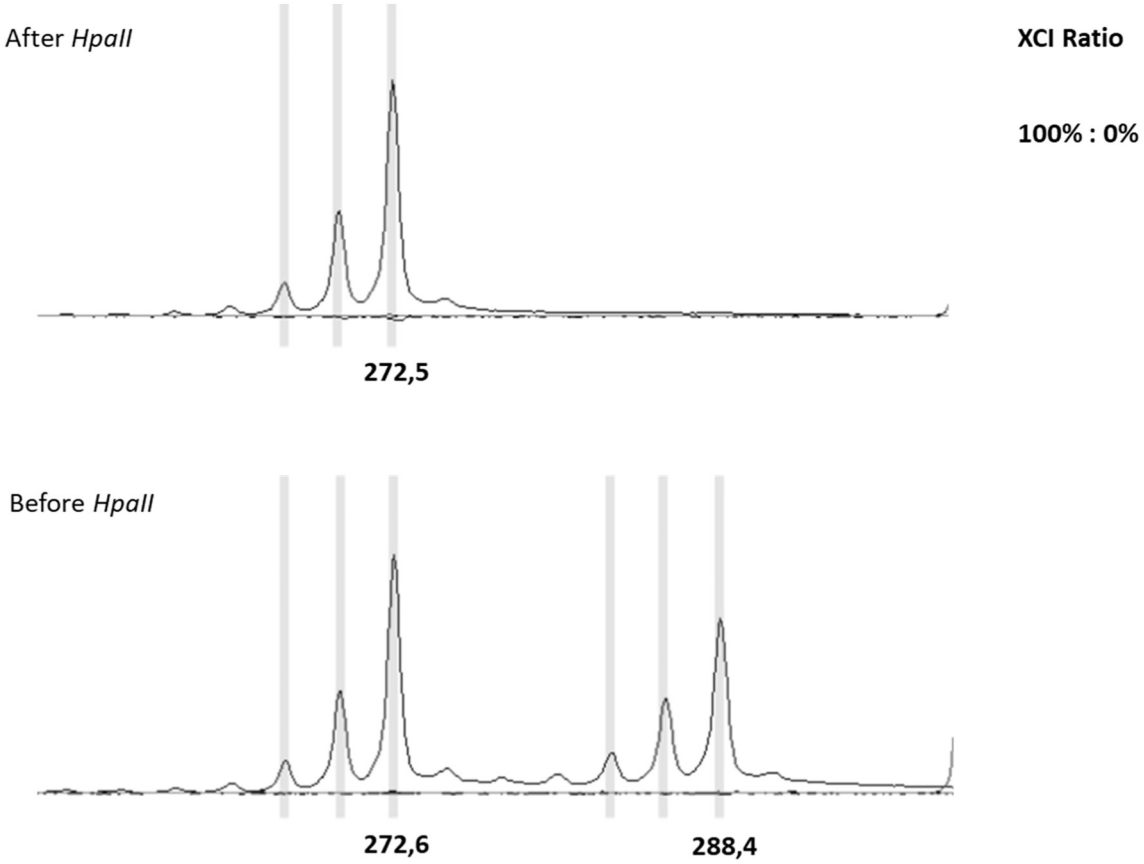

Fig. 2. XCI analysis based on the analysis of methylation specificity of *Hpa*II restriction enzyme at polymorphic short tandem repeats in the AR gene in a patient with extreme XCI skewing.

Turner's syndrome (monosomy X) and a be a carrier of a novel missense p.Cys2040Arg *F8* mutation.

3.7. 46,XY androgen insensitivity syndrome

Another patient (FVIII activity < 1%) was shown to have a 46,XY karyotype androgen insensitivity syndrome caused by a novel *AR* mutation (NM_000044.3:c.2319-2A > G splice site acceptor mutation) combined with *F8* gene intron 22 inversion. This is the only patient with factor VIII inhibitor in our study. Prior to the study this 55-year-old nullipara was not aware of the 46,XY karyotype and did not consent for further gynecological and endocrine work-up once received the genetic diagnosis. We also performed genetic analysis in the brother of the proband, who also suffers from severe hemophilia A, and this allowed us to conclude this AR variant is a de novo mutation (the genotype of the mother can be established based on the data from offspring, Fig. 1). There are already some reports of hemophilia in females with 46,XY karyotype but most of them date back several decades [15–17].

3.8. Compound heterozygosity

One patient (FVIII activity 4%) presented with 2 pathogenic *F8* point mutations: novel nonsense p.Leu2170CysfsTer16 and previously reported missense p.Tyr1802Cys.

3.9. Skewed X chromosome inactivation combined with an F8 mutation is causative in most cases

In 10 females hemophilia A phenotype could be ascribed to a mutation/rearrangement in *F8* combined with abnormally skewed X chromosome inactivation. This is presented in Table 3. This must be acknowledged that XCI was assessed from peripheral blood leukocytes, as FVIII producing tissue is not available for practical and ethical reasons. Fig. 2 presents an example of XCI analysis with extreme skewing. The patient with 0.5% FVIII activity and p.Trp604Cys mutation is the only one receiving FVIII prophylaxis in our cohort (because of bleeding to a target joint) (Fig. 2).

4. Discussion and conclusion

We provide a first molecular epidemiologic data from a nationwide survey of the clinical features and genetic etiology in Polish women with hemophilia A. Regarding *F8* defects, similar to reports from other ethnic groups there is a high prevalence of private mutations, these including 3 novel disease-coding *F8* variants, so far not found in males. It is possible to speculate on the phenotype of the 3 novel mutations. Asn1460LysfsTer2 was also found in proband's son suffering from severe hemophilia, p.Cys2040Arg likely leads to phenotype of moderate hemophilia (FVIII activity of 4,5% in Turner syndrome patient), Ser226Asn seem to lead to severe or moderate phenotype (FVIII activity of 3% in a female with extremely skewed XCI). Most importantly, we identified a wide and frequently surprising range of defects leading to the phenotype of hemophilia A in females. Of particular interest was a patient with a 46,XY karyotype and a novel *AR* mutation leading to androgen insensitivity syndrome. In general, the molecular events leading to female hemophilia, especially moderate/severe must be complex and may be also causative for other morbidity. Full description of the frequently surprising comorbidities and the association of hemophilia-causative genomic defects with phenotypes broader than the coagulation defect may facilitate proper management (as exemplified by a contiguous gene syndrome associated with hemophilia A that we

previously described) [4–6].

This level of genomic complexity is similar in our recent report of females with hemophilia B in Poland although unlike in hemophilia B, in this cohort of females with hemophilia A we identified no chromosomal translocations, which seem to be causative for a proportion of females with hemophilia B [12]. The genetic diagnosis of female hemophilia A may have impact on clinical treatment as well as genetic counselling and the emerging six molecular patterns we describe plus chromosomal translocations and gross deletions form a catalogue of genomic events to be investigated. Skewed XCI as a cause for X-linked congenital disorders in females should be interpreted with caution following exhaustive genetic work-up.

Author contribution

Szymon Janczar and Wojciech Mlynarski initialized the study, analyzed data and wrote the manuscript. Katarzyna Bąbol-Pokora, Iza Jatczak-Pawlik, Joanna Taha and Edyta Odnoczko, performed the laboratory studies and analyzed data. The remaining authors recruited study subjects, provided clinical data and reviewed the manuscript.

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Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2020.05.041.

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